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**13. ABSTRACT** ***(Maximum 200 Words)***

Taxol has been approved by the FDA for the treatment of breast, ovarian and lung carcinomas, and has proven to be an important drug. Its antitumor activity is derived primarily from its ability to bind to β-tubulin in the microtubule, thereby stabilizing the polymer and inhibiting cell division. However, development of Taxol resistance represents a major obstacle for the effective treatment of cancer. β-tubulin gene mutations have been identified as a strong predictor of the response to Taxol in patients. Resistant cells isolated in the presence of Taxol, and other recently discovered microtubule-stabilizing agents such a epothilone, eleutherobin, and discodermolide, have been shown to carry specific mutations in β-tubulin. However, isolation of spontaneous mutations can be a long and tedious process. In this study, I plan to identify resistant mutations in β-tubulin by randomly mutating human β-tubulin genes, and by selecting the mutant-expressing cells in the presence of Taxol. Taxol resistant cells will be tested for sensitivity to various tubulin drugs. My specific aim is to correlate each mutation with a specific drug response profile. A comprehensive database with mutation information and drug-sensitivity profile will be a valuable tool. It will help to rapidly locate the most effective drugs for cancer patients with specific tubulin mutations. In addition, structural information obtained from mutations at specific sites in β-tubulin will benefit the rational design of new anti-tumor drugs.

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## Table of Contents

**Introduction:**

Taxol has been approved by the FDA for the treatment of breast, ovarian and lung carcinomas, and has proven to be an important drug. Its antitumor activity is derived primarily from its ability to bind to β-tubulin in the microtubule, thereby stabilizing the polymer and inhibiting cell division. However, development of Taxol resistance represents a major obstacle for the effective treatment of cancer. Previously β-tubulin gene mutations were proposed to be a predictor of the response to Taxol in patients [1]. Based on this observation, I submitted my concept proposal "Relationship between ß-Tubulin Mutations and Taxol Resistance in Breast Cancer Cells" in April of 2000. The goal in that proposal was to establish the correlation between ß-mutations with a specific response profile to Taxol-like drugs.

Since the submission and approval of the proposal, accumulating evidences indicated that cell responses to Taxol-like drugs could be complicated. For example, Taxol may inhibit cell proliferation without blocking cells at mitosis [2]. Therefore we have to define drug responses first, prior to correlating β-tubulin mutations with a specific drug response profile. This idea also came at a time when the initial observation on tubulin mutations in cancer patients failed to be repeated. In a group of breast cancer patients treated with Taxol, no ß-mutations were observed [3].

Under the support of this concept award, I studied the mechanism of action of Taxol-like drugs at low concentrations. Although the experiments were not exactly the same as those in the proposal, they are very related and pertinent to the improvement of chemotherapy in breast cancer.

**Body:**

## 1. Induction of an aneuploid population by microtubule-stabilizing agents.

A variety of antimitotic agents interact in the tubulin/microtubule system, a validated target for cancer chemotherapy. Taxol, the epothilones and discodermolide have a binding site in β-tubulin in the microtubule polymer, and stabilize microtubules promoting the formation of microtubule bundles. In contrast, vinblastine, colchicine and nocodazole bind primarily to αβ–tubulin dimers and prevent assembly of microtubules. The effects of these drugs on cell cycle profiles in human non-small cell lung carcinoma A549 cells were determined using flow cytometry. A large population of aneuploid cells, close to the diploid 2N peak, was observed on cell cycle analysis after incubation of the cells with low concentrations of drugs (Fig 1). All three microtubule stabilizing agents, Taxol, EpoB and discodermolide induced aneuploid populations in A549 cells. In contrast to the stabilizing drugs, three microtubule destabilizing agents including vinblastine, colchicine and nocodazole did not induce a large population of aneuploid cells in the absence of mitotic block (Fig 2, appended paper).

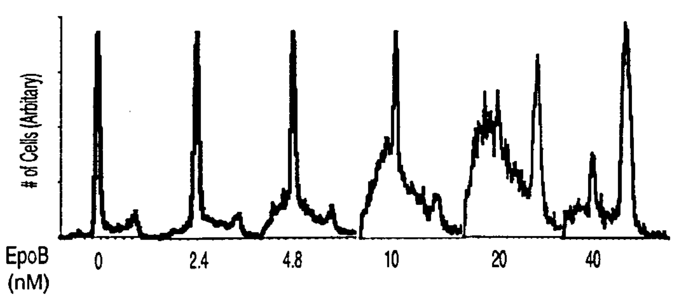


*Fig 1. A: A549 cells were incubated for 18 h with increasing concentrations (nM) of EpoB. The cells were fixed and stained with propidium iodide, and analyzed by flow cytometry.*

## 2. Aberrant spindles and cell division.

The aneuploid population disappeared at higher concentrations of the stabilizing drugs when the cells were blocked at mitosis (Fig 1), suggesting that induction of aneuploid cells requires mitotic cell cycle progression. Mitoses were examined by fluorescent microscopy and four kinds of spindle structures were noticed in different cells prior to division (Fig. 3, appended paper). Multipolar spindles (>2), with abnormal chromosome alignments, were observed in cells treated

with the stabilizing drugs. Mitotic cells with multipolar spindles resulted in aberrant cell divisions. Approximately 15% of the telophase cells, after incubation with Taxol or EpoB, produced 3 daughter cells connected by multiple midbodies, while the remaining 85% of the telophase cells produced two daughter cells. With discodermolide, 25% of the telophase cells produced tripolar cell division. In contrast, no tripolar cell division was observed in cells treated with the destabilizing drugs. The results suggest that a significant percentage of mitotic cells contained multipolar spindles that led to aberrant cell division and produced aneuploid G1 cells (Fig 3, appended paper).

### 3. Apoptosis after aberrant mitosis

Cells resulting from aberrant mitosis have variable DNA content, are of different sizes, and have a mixed number of nuclei [4]. These cells are arrested at G1 phase and unable to proliferate as a result of the activation of G1 checkpoint genes p53 and p21 [4]. To explore the mechanism of cell death of these aneuploid cells, gene expression in A549 cells treated with 10 nM epothilone, 8 and 45 nM Taxol, is being examined using Affymetrix microarray chips. One group of genes are significantly up-regulated by 8 nM Taxol and 10 nM epothilone B, but less significantly by a higher concnetration of Taxol (45 nM) that results in a mitotic block (Table 1). Many of the genes falling in this group are known p53 dependent, stress response genes [5]. It is hypothesized that aneuploid cells may inherit DNA damage after aberrant mitosis. Induction of proapoptotic genes including CD95 and Bax could be the uderlining mechanism of cytotoxicity of these drugs at low concentrations.

### 4. Aneuploidy and Drug Sensitivity

If aneuploid cells die of apoptosis, drug sensitivity for microtubule stabilizing agents should correspond to the induction of aneuploid G1 cells. The drug concentrations required for 50% inhibition of cell growth after a 72 h incubation ($IC_{50}$) are compared with the concentrations of drug required for initiation of aneuploidy and for the induction of mitotic arrest (Table 2). $IC_{50}$ values for Taxol and discodermolide were closer to the concentrations of drug required for initiation of aneuploidy than for the induction of mitotic arrest. Interestingly, the $IC_{50}$ for epothilone B was even lower than the drug concentration needed for half maximal induction of aneuploidy.

*Table 1. Gene expression in cells treated with epothilone B and Taxol compared to that in control cells*

| Accession # | Gene Name | Epo10 | TX8 | TX45 |
|---|---|---|---|---|
| M11717 | Heat shock protein (hsp 70) | 1.9 | 1.64 | 0.87 |
| J03826 | Adrenodoxin reductase | 1.74 | 2 | 1.07 |
| AB020645 | KIAA0838 protein, GLS Glutaminase | 1.83 | 2 | 1.71 |
| AF010309 | Pig3 (PIG3) | 1.62 | 1.85 | 1.36 |
| W28438 | unknown | 1.78 | 1.91 | 1.45 |
| X63717 | APO-1 cell surface antigen /cds | 1.39 | 1.86 | 1.43 |
| AF038844 | MKP-1 like protein tyrosine phosphatase | 1.75 | 1.97 | 1.67 |
| X13839 | Vascular smooth muscle alpha-actin | 1.85 | 2.62 | 1.11 |
| U72649 | BTG2 (BTG2) mRNA | 2.24 | 2.91 | 1.55 |
| AL031432 | Clone 465N24 on chromosome 1p35.1-36.13 | 1.87 | 2.02 | 1 |
| L22475 | Human Bax gamma mRNA, complete cds | 2.06 | 2.41 | 1.08 |
| U03106 | Human wild-type p53 activated fragment-1 (WAF1) | 2.82 | 2.59 | 2.01 |
| U19599 | Human (BAX delta) mRNA, complete cds | 2.26 | 2.31 | 0.98 |
| M60974 | Growth arrest and DNA-damage-inducible protein gadd45 | 1.48 | 1.86 | 1.71 |
| AB000584 | TGF-beta superfamily protein, complete cds | 2.48 | 2.9 | 2.19 |
| U48807 | Human MAP kinase phosphatase (MKP-2) | 1.75 | 1.81 | 1.33 |
| X83490 | Fas/Apo-1 (clone pCRTM11-Fasdelta(3,4)) | 1.72 | 2.13 | 1.43 |
| U18300 | Damage-specific DNA binding protein p48 subunit (DDB2) | 1.86 | 1.82 | 0.97 |

*Table 2. Comparision of cytotoxicity with aneuploid cell production and mitotic block*

| Drugs (nM) | $IC_{50}$ | Aneuploid | Mitotic block |
|---|---|---|---|
| Taxol | 3.4 | 2.2 | 18.5 |
| EpoB | 0.57 | 3.3 | 28.3 |
| Discodermolide | 9.5 | 12.1 | 49.0 |

To further examine the significance of aneuploidy induction on EpoB sensitivity, an EpoB resistant cell line, A549.EpoB40, was examined. This cell line has a β-tubulin mutation at amino acid 292 and is 95-fold resistant to EpoB compared to the parental cells [6]. Although a mitotic block did occur in response to increasing concentrations of EpoB, there was a large decrease in the aneuploid population compared to that in the drug-sensitive cells (Fig 2A). In contrast, the A549.EpoB40 resistant cell line that does not demonstrate cross resistance to discodermolide [6], exhibited a large increase in the aneuploid population in response to discodermolide (Fig 2B).

These results indicate that EpoB drug resistance is partly determined by inhibition of aneuploid induction.

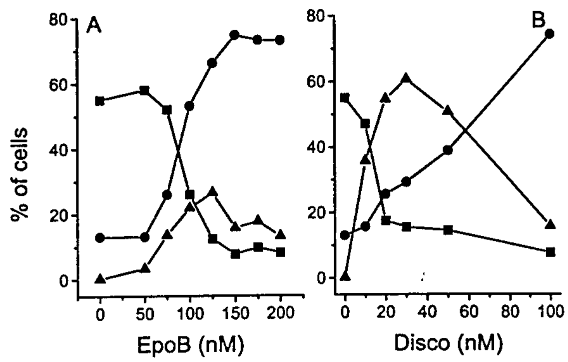


Fig. 2. *Dose response curves of the aneuploidy (▲) compared to the G1 (■) and G2/M (●) euploidy populations in A549.EpoB40 cells. Cells were incubated with increasing concentrations (nM) of EpoB (A) or discodermolide (B) for 18 h. Cell cycle profiles were measured as in Fig 1.*

## Key Research Accomplishments:

1. Microtubule stabilizing agents induce an aneuploid population prior to mitotic arrest.
2. Aneuploid cells originate from aberrant mitosis.
3. Aneuploid cells are arrested at G1 due to activation of p53 and p21, and will die of apoptosis.
4. Induction of aneuploidy determines sensitivity of cells to MT stabilizing agents.

## Reportable Outcomes:

Part of this study was published in the April 1, 2002 issue of Cancer Research. A reprint of this paper, that acknowledges the support from The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0674, is attached.

## Conclusions:

This study helps us to better understand the mechanisms of action of Taxol, an approved drug for the treatment of breast cancer. The results from this study suggest that Taxol-like drugs have multiple mechanisms of action depending on the drug concentration. Although both microtubule stabilizing and destabilizing drugs induce mitotic block, only the former may induce aberrant mitosis through multipolar spindle formation that results in aneuploid populations of cells. After aberrant mitosis, cells are arrested at G1 and likely die of apoptosis. The latter determines the sensitivity of cells to Taxol-like drugs. It is possible that Taxol may be effective when applied to cancer patients at lower concentrations than the current dosage used in the clinic.

Tumor cells normally display a marked genetic instability due to amplification of centrosomes and formation of multipolar spindles. As evidenced by a high level of apoptosis, DNA replication in these cells operates near the error threshold for cell viability. Therefore tumor cells cannot sustain further disruption of its genome from increased aberrant mitosis due to microtubule stabilizing drugs. However, if microtubule- stabilizing agents initiate multipolar spindles and aberrant mitosis in normal somatic cells, they could destabilize the normal cell

genome as well, thereby being potentially mutagenic. As a successful anticancer agent, Taxol may possess partial selectivity *in vivo.* It would be of interest to investigate if there is a different mitotic response between normal and cancer cells in response to low concentrations of microtubule stabilizing drugs.

**References:**


1. Monzo, M., R. Rosell, et al. (1999). "Paclitaxel resistance in non-small-cell lung cancer associated with beta-tubulin gene mutations." J Clin Oncol 17(6): 1786-93.

2. Giannakakou, P., Robey, R., Fojo, T., and Blagosklonny, M. V. Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity, Oncogene. *20:* 3806-13, 2001.

3. Rafael Rosell. Progress in pharmacogenomic strategies for developing customized chemotherapy: tubulin mutations and ERCC1 expression in the context of genotypic international lung trial (GILT). America Association for Cancer Research Meeting, 2002, CA.

4. Torres, K. and Horwitz, S. B. Mechanisms of Taxol-induced cell death are oncentration dependent, Cancer Res. *58:* 3620-6, 1998. 1.

5. Yu, J., Zhang, L., Hwang, P. M., Rago, C., Kinzler, K. W., and Vogelstein, B. Identification and classification of p53-regulated genes, Proc Natl Acad Sci U S A. *96:* 14517-22., 1999.

6. He, L., Yang, C.-P. H., and Horwitz, S. B. Mutations in β-tubulin map to domains involved in regulation of microtubule stability in epothilone-resistant cell lines, Molecular Cancer Therapeutics. *1:* 3-10, 2001.


**Appendices:**

1. Chen JG and Horwitz SB (2002). Differential Mitotic Responses to Microtubule Stabilizing and Destabilizing Agents. *Cancer Research. 62: 1935-1938.*

[CANCER RESEARCH 62, 1935–1938, April 1, 2002]

*Advances in Brief*

# Differential Mitotic Responses to Microtubule-stabilizing and -destabilizing Drugs[1]

**Jie-Guang Chen and Susan Band Horwitz[2]**

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## Abstract

**Although microtubule interacting agents inhibit spindle dynamics, thereby leading to a block in mitosis, we report that low concentrations of these drugs result in differential mitotic effects. Microtubule-stabilizing agents including Taxol, epothilone B, and discodermolide produce aneuploid populations of A549 cells in the absence of a mitotic block. Such aneuploid populations are diminished in an epothilone B-resistant cell line. In contrast, microtubule-destabilizing agents like colchicine, nocodazole, and vinblastine are unable to initiate aneuploidy. The aneuploid cells result from aberrant mitosis as multipolar spindles are induced by the stabilizing drugs, but not by destabilizing agents. The results suggest that the mechanism underlying aberrant mitosis may not be the same as that responsible for mitotic block, and that the former determines the sensitivity of cells to Taxol-like drugs.**

## Introduction

A variety of antimitotic agents interact in the tubulin/microtubule system, a validated target for cancer chemotherapy. Taxol, the epothilones, and discodermolide have a binding site in $\beta$-tubulin in the microtubule polymer, and stabilize microtubules promoting the formation of microtubule bundles (1–3). In contrast, vinblastine, colchicine, and nocodazole bind primarily to $\alpha\beta$–tubulin dimers and prevent assembly of microtubules. However, low concentrations of both Taxol and vinblastine suppress growing and shortening at the ends of microtubules and appear to block mitosis by dynamically stabilizing spindle microtubules (4, 5).

Cell death occurs in cancer cells treated with Taxol (6), and the question arises as to the relationship between mitotic block and cell death. Although Taxol-induced apoptosis may occur after a prior mitotic arrest (7), it could also be induced independently of $G_2$-M arrest (8). Previous studies with lung carcinoma A549 cells found that low concentrations of Taxol inhibited cell proliferation without blocking cells at mitosis (9) and induced a large population of hypodiploid cells seen close to the $G_1$ peak (10). It was speculated that the aneuploid cells might originate from aberrant mitosis (10), although the mechanism remained to be determined.

Because suppression of spindle dynamics by low concentrations of drugs is a common mechanism responsible for mitotic block, induced by both microtubule-stabilizing and -destabilizing agents (4, 5), we questioned whether other microtubule-interacting drugs would, like Taxol, induce aneuploid cells without blocking cells at mitosis. The ability of six antimitotic drugs to initiate aberrant mitosis and to induce an aneuploid population of A549 cells has been analyzed. Our results indicate that the stabilizing drugs, but not the destabilizing agents, induce aberrant mitosis through multipolar spindle formation that results in aneuploid populations of cells.

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## Materials and Methods

**Cell Culture and Reagents.** Human non-small cell lung carcinoma cells, A549, were cultured as described previously (10). A549.EpoB40, an EpoB[3]-resistant cell line, was isolated from A549 cells in our laboratory, and is maintained in 40 nM EpoB (11). Monolayer cultures were grown in 7% $CO_2$ and passaged at intervals of 4 days. Fluorescent dyes rhodamine-phalloidin and Alexa 488-conjugated antimouse IgG were from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co.

**Flow Cytometry.** Distribution of DNA content in A549 cells treated with microtubule interacting drugs was determined by flow cytometry (10). Briefly, cells treated with drugs were fixed in 70% ethanol and resuspended in PBS containing PI and DNase-free RNase A. Forward and orthogonal scatter lights, as well as red fluorescence were analyzed by fluorescence-activated cell sorting. A dual parameter dot plot of the integrated area verses the width of the fluorescence pulse was displayed to exclude cell aggregates from cell counting and analysis. The histogram of DNA distribution was modeled as a sum of $G_1$ (Fig. 1*B 2N*), $G_2$-M (Fig. 1*B*, *4N*), S phase, and an aneuploid population close to the $G_1$ peak, by using ModFitLT software (Fig. 1*B*).

**Immunofluorescence Microscopy.** After growth for 48 h on coverslips, subconfluent A549 cells were incubated with drugs at the indicated concentrations for 18 h. Cells were made permeable and fixed (10). After blocking, cells were incubated with mouse monoclonal $\alpha$-tubulin antibody for 1 h at 37°C. The secondary antibody, Alex 488-conjugated goat antimouse IgG, was added to the cells for 1 h, in the presence of rhodamine-phalloidins for actin staining. Chromosomes were stained with 1 $\mu$g/ml DAPI in PBS. After washing with PBS, the slides were mounted and sealed. Fluorescent images were acquired with an Olympus high-resolution microscope, connected to a CCD camera. Tricolor images were merged using Photoshop 5.5. Approximately 100 mitotic cells were counted to calculate the number of spindles per cell.

## Results and Discussion

**Induction of an Aneuploid Population by Microtubule-stabilizing Agents.** It was previously noted that treatment of human lung carcinoma A549 cells with low concentrations of Taxol (nM) resulted in a hypodiploid population of cells in the absence of a $G_2$-M block (10). This phenomenon was examined with two other microtubule-stabilizing agents, EpoB and discodermolide. A large population of hypodiploid cells, close to the diploid 2N peak, was observed on cell cycle analysis after incubation of A549 cells with increasing concentrations of EpoB for 18 h. In addition, a small shoulder was seen to the right of the 2N DNA (Fig. 1*A*). The hypodiploid population and the shoulder, taken together, can be best fitted by a symmetric distribution near the $G_1$ peak, in addition to the standard distribution of the cell cycle (Fig. 1*B*). A dose-response curve is shown in Fig. 2 that compares induction of the aneuploid population with the $G_1$ and $G_2$-M phases of the normal cell cycle for six microtubule interacting agents. If the hypodiploid portion alone were plotted instead of the whole aneuploid population, the dose-response curve would be the same, although with lower percentages of cells.

All three microtubule-stabilizing agents, Taxol, EpoB, and discodermolide induced aneuploid populations in A549 cells. The aneu-

[3] The abbreviations used are: EpoB, epothilone B; PI, propidium iodide; DAPI, 4′,6-diamidino-2-phenylindole.

ploid population was most apparent before an increase in the $G_2$-M population. This population, including both the hypodiploid cells and the cells represented by the right shoulder of the 2N peak, disappeared at higher concentrations of the stabilizing drugs when the cells were blocked at mitosis (Fig. 2). Treatment with 0.2 mM $H_2O_2$ or 2 $\mu$M geldanamycin, both of which caused mitotic arrest, together with 10 nM EpoB for 18 h, decreased the EpoB-induced aneuploid population (data not shown). Induction of aneuploid cells evidently requires mitotic cell cycle progression, suggesting that aneuploid populations, amid normal $G_1$ cells, originate from aberrant mitosis (12). In contrast to the stabilizing drugs, three microtubule-destabilizing agents including vinblastine, colchicine, and nocodazole did not induce a large population of aneuploid cells in the absence of mitotic block (Fig. 2).

**Aberrant Spindles and Cell Division.** Mitoses were examined by fluorescent microscopy, and four kinds of spindle structures were

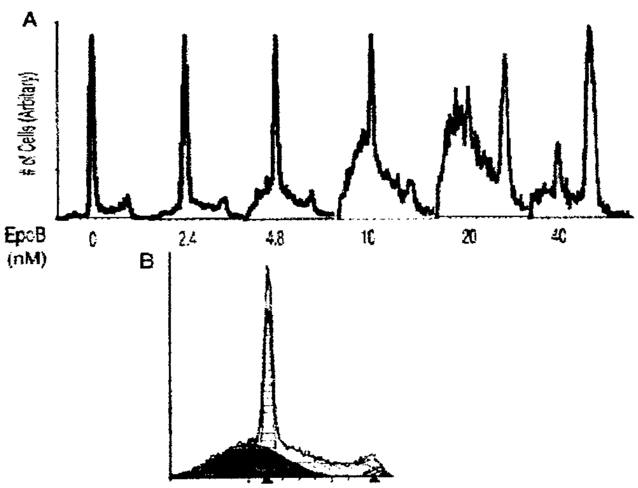


Fig. 1. *A*, A549 cells were incubated for 18 h with increasing concentrations (nM) of EpoB. The cells were fixed and stained with PI, and analyzed by flow cytometry. *B*, histogram of DNA distribution in cells treated with 4.8 nM EpoB was modeled as a sum of $G_1$ (*2N*, ▤), $G_2$-M (*4N*, ▨), S-phase (▧), and an aneuploid population (■).

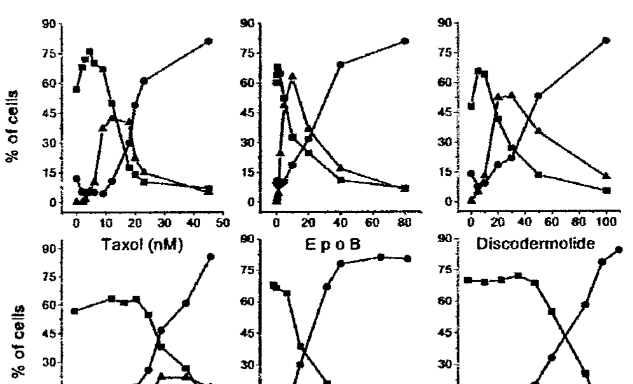


Fig. 2. Dose-response curves of the aneuploidy (▲) compared with the $G_1$ (■) and $G_2$-M (●) euploidy populations. A549 cells were incubated for 18 h with Taxol, EpoB, discodermolide, vinblastine, colchicine, or nocodazole (nM). Cells were stained with PI and analyzed by flow cytometry as in Fig. 1. The population data were calculated by fitting the DNA histogram to models using ModFitLT software. Data are from one representative experiment that has been repeated at least once. Peak aneuploidy induced by 12 nM Taxol, 10 nM EpoB, or 20 nM discodermolide was 45.5% ± 4.4, 62.4% ± 2.8, or 52.5% ± 0.5 ($n$ = 2–3), respectively.

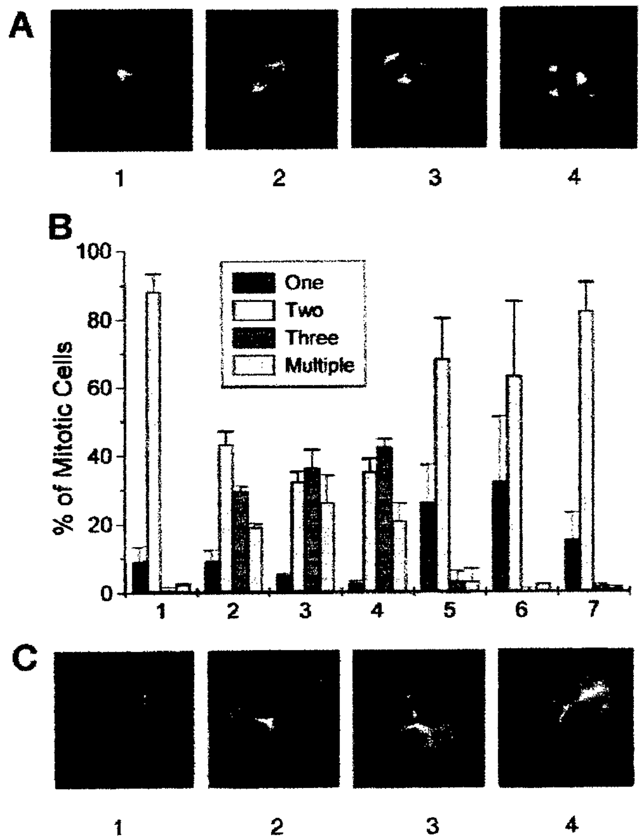


Fig. 3. *A*, A549 cells treated with 4.8 nM EpoB for 18 h were stained with $\alpha$-tubulin antibody (*green*, microtubules), DAPI (*blue*, chromosomes) and rhodamine-phalloidins (*red*, actin fibers). Four representative mitotic cells with different spindle structures and chromosome alignments were visualized by fluorescent microscopy. *B*, percentages of mitotic cells with one, two, three, or more spindles after drug treatment. Untreated cells (*columns 1*) and cells treated with 8 nM Taxol (*columns 2*), 4.8 nM EpoB (*columns 3*), 10 nM discodermolide (*columns 4*), 10 nM colchicine (*columns 5*), 65 nM nocodazole (*columns 6*), or 7 nM vinblastine (*columns 7*) were stained and visualized as above. Data are expressed as means ± SD from two experiments. *C*, examples of aberrant cell division after treatment with 4.8 nM EpoB for 18 h. *Panel 1*, cleavage furrow occurs in a cell that will give rise to two daughter cells with unequal DNA; *panels 2 and 3*, aberrant cell division results in three daughter cells of different sizes; *panel 4*, four cells, of different sizes and DNA content, are connected by multiple midbodies.

observed in different cells before division (Fig. 3*A*). Some cells (Fig. 3, *A.1*) contained a ring of condensed chromosomes with a monopolar spindle pole in the center. Bipolar spindles (Fig. 3, *A.2*) were present not only in normal cells, but also in drug-treated cells. Lagging chromosomes close to spindle poles were occasionally found in drug-treated metaphase cells. In the absence of proper spindle attachment, lagging chromosomes may activate a spindle checkpoint and block anaphase transition (13). Tripolar (Fig. 3, *A.3*) and quadripolar (Fig. 3, *A.4*) spindles, with abnormal chromosome alignments, also were seen in cells treated with the stabilizing drugs. Multipolar spindles have been observed previously in cells treated with taxanes (14, 15). For a detailed view of bipolar, tripolar, and tetrapolar spindles and their associated chromosomes in EpoB-treated cells, three-dimensional movies have been generated from confocal fluorescent microscopy and are available from the authors or on the Internet.[4] (QuickTime software is required to view the files.)

The spindle structures were compared at those drug concentrations at which the $G_1$ population was initially decreased (Fig. 2). Multipolar

[4] Internet address: www.aecom.yu.edu/aif/users/s_horwitz/mitosis.htm.

spindles (>2) were noted only in cells treated with the stabilizing drugs, but not in cells treated with the destabilizing agents (Fig. 3*B*). For cells treated with the destabilizing drugs, such as *HeLa* cells incubated with *Vinca* alkaloids (16), there was an increase in the incidence of mitotic cells with monopolar spindles. It is important to point out, however, that cells containing the same number of spindles may have subtle differences resulting from different drug treatments. For example, monopolar spindles in the cells treated with the destabilizing drugs displayed distinct astral microtubules irradiating from a nucleation center (data not shown). In contrast, cells treated with the stabilizing agents have a condensed, ball-shaped microtubule center (Fig. 3, *A.1*).

As long as chromosomes can congress and align, although they are distorted (Fig. 3, *A.3* and *A.4*), the presence of multipolar spindles does not alert the checkpoint controlling the exit of mitosis (17). Mitotic cells with multipolar spindles resulted in aberrant cell divisions. A few cells gave rise to unequal division (Fig. 3, *C.1*), which would produce not only hypodiploid cells but also $G_1$ cells with more than 2N DNA. Tripolar cell divisions were significantly increased in cells treated with microtubule-stabilizing drugs compared with control cells (Fig. 3, *C.2* and *C.3*). At the drug concentrations used in Fig. 3*B*, ~15% of the telophase cells, after incubation with Taxol or EpoB, produced three daughter cells connected by multiple midbodies, whereas the remaining 85% of the telophase cells produced two daughter cells. With discodermolide, 25% of the telophase cells produced tripolar cell division. In contrast, no tripolar cell division was observed in cells treated with the destabilizing drugs at the concentrations indicated in Fig. 3*B*, nor in cells treated with 9 and 11 nM of vinblastine (data not shown). Both tripolar cell division and multipolar spindles occurred only in cells treated with the stabilizing drugs. A higher percentage of tripolar cell division might have been expected if all mitotic cells with tripolar spindles had progressed to tripolar cell division. However, the number of tripolar cells may be underestimated if one cell was separated early from the two other connected cells. Our results suggest that a significant percentage of mitotic cells contained multipolar spindles that led to aberrant cell division and produced aneuploid $G_1$ cells. Multipolar spindles have been observed in many tumor cells and are responsible for the induction of aneuploidy (18).

**Aneuploidy and Drug Sensitivity.** A significant aneuploid population was induced by treatment with 8 nM Taxol for 18 h (Fig. 2). In response to an additional treatment with 36 nM Taxol that blocked cells in mitosis, the accumulation of cells at the $G_2$-M phase was significantly slower compared with cells without a preincubation (data not shown). This suggests that part of the cells were not cycling after preincubation with Taxol. Taxol may have activated the $G_1$ checkpoint genes *p53* and *p21* (9), and induced a $G_1$ block in cells after division (19). Consistent with previous observations in Taxol-treated cells (20, 21), drug sensitivity for microtubule-stabilizing agents corresponds to the induction of aneuploid $G_1$ cells. The drug concentrations required for 50% inhibition of cell growth after a 72-h incubation ($IC_{50}$) with Taxol and discodermolide were 3.4 and 9.5 nM, respectively. These concentrations were closer to the concentrations of drug required for the initiation of aneuploidy than for the induction of mitotic arrest (Fig. 2). Interestingly, EpoB had an $IC_{50}$ value of 0.57 nM, which was even lower than the drug concentration (3.3 nM) needed for one-half maximal induction of aneuploidy (Fig. 2). This suggests that induction of an aneuploid population may not fully account for the EpoB cytotoxicity, and that other mechanisms may be involved. Alternatively, more accurate methods such as fluorescence *in situ* hybridization may be required to measure aneuploidy induction by EpoB at concentrations close to its $IC_{50}$ value.

To further examine the significance of aneuploidy induction on EpoB sensitivity, an EpoB-resistant cell line, A549.EpoB40, was examined. This cell line has a $\beta$-tubulin mutation at amino acid 292 and is 95-fold resistant to EpoB compared with the parental cells (11). Although a mitotic block did occur in response to increasing concentrations of EpoB, there was a large decrease in the aneuploid population compared with that in the drug-sensitive cells (compare Fig. 4*A* with EpoB in Fig. 2). In contrast, the A549.EpoB40 resistant cell line that does not demonstrate cross-resistance to discodermolide (11), exhibited a large increase in the aneuploid population in response to discodermolide (Fig. 4*B*). These results indicate that EpoB drug resistance is partly determined by the inhibition of aneuploid induction.

Our observations indicate that aneuploidy is a result of aberrant mitosis and contributes to cell death in drug-sensitive cells. Aberrant mitosis may confer a selective killing of malignant cells by microtubule-stabilizing drugs, because induction of aneuploidy through aberrant mitosis creates chromosomal breakage, interchromosomal concatenation, and a lethal genetic imbalance (22). Tumor cells normally display a marked genetic instability caused by amplification of centrosomes and formation of multipolar spindles (18). As evidenced by a high level of apoptosis, DNA replication in these cells operates near the error threshold for cell viability (23). Therefore, a tumor cell cannot sustain further disruption of its genome from increased aberrant mitosis attributable to microtubule-stabilizing drugs. However, if microtubule-stabilizing agents initiate multipolar spindles and aberrant mitosis in normal somatic cells, they could destabilize the normal cell genome as well, thereby being potentially mutagenic. It would be of interest to investigate whether different mitotic responses occur in normal and cancer cells in response to low concentrations of microtubule-stabilizing drugs.

In contrast to mitotic arrest, aberrant mitosis was induced only by microtubule-stabilizing drugs and not by destabilizing agents. This suggests that suppressing spindle dynamics cannot fully account for aberrant mitosis. In addition, a complete mitotic block, but not aneuploidy, was induced by higher concentrations of EpoB in A549.EpoB40-resistant cells (Fig. 4). This further supports the concept that the mechanisms responsible for mitotic block and aberrant mitosis may not be the same. Interference with the function of centrosomes may contribute to aberrant mitosis (15), because Taxol has been reported to preferentially bind to centrosomes (24). Preliminary studies using antibodies against $\gamma$-tubulin and pericentrin found two major centrosomes and additional minor ones in some aberrant mitotic cells after treatment with 4.8 nM EpoB (data not shown). Although centrosomes may not be required for the function of spindle poles, the frequency of midbody formation and successful division is higher when centrosomes are present (25). Centrosome amplification has been associated with induction of multipolar spindles and aneu-

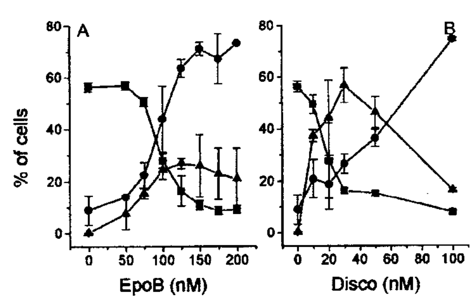


Fig. 4. A549.EpoB40 cells were grown in the absence of drug for 2 weeks before the experiment. The cells were incubated with increasing concentrations (nM) of EpoB (*A*) or discodermolide (*B*) for 18 h. Cell cycle profiles were measured as in Fig. 1, calculated and represented as in Fig. 2. Data are expressed as means ± SD from two experiments.

ploidy in cancer cells (18). Further research is needed to elucidate the molecular mechanisms of multipolar spindle induction by the microtubule-stabilizing drugs.

In summary, we have demonstrated that the response to different microtubule-interacting agents can be distinct. The occurrence of aberrant mitosis and aneuploidy is dependent on both the mechanism of drug action and the concentration of drug. The sensitivity of cells to microtubule-stabilizing drugs is mainly the result of aberrant mitosis and not mitotic block.

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## References

1. Schiff, P. B., and Horwitz, S. B. Taxol stabilizes microtubules in mouse fibroblast cells. Proc. Natl. Acad. Sci. USA, *77:* 1561–1565, 1980.
2. Bollag, D. M., McQueney, P. A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., Goetz, M., Lazarides, E., and Woods, C. M. Epothilones, a new class of microtubule-stabilizing agents with a Taxol-like mechanism of action. Cancer Res., *55:* 2325–2333, 1995.
3. ter Haar, E., Kowalski, R. J., Hamel, E., Lin, C. M., Longley, R. E., Gunasekera, S. P., Rosenkranz, H. S., and Day, B. W. Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than Taxol. Biochemistry, *35:* 243–250, 1996.
4. Jordan, M. A., Toso, R. J., Thrower, D., and Wilson, L. Mechanism of mitotic block and inhibition of cell proliferation by Taxol at low concentrations. Proc. Natl. Acad. Sci. USA, *90:* 9552–9556, 1993.
5. Jordan, M. A., Thrower, D., and Wilson, L. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. J Cell Sci., *102:* 401–416, 1992.
6. Moos, P. J., and Fitzpatrick, F. A. Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. Cell Growth Differ., *9:* 687–697, 1998.
7. Woods, C. M., Zhu, J., McQueney, P. A., Bollag, D., and Lazarides, E. Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway. Mol. Med., *1:* 506–526, 1995.
8. Lieu, C. H., Chang, Y. N., and Lai, Y. K. Dual cytotoxic mechanisms of submicromolar Taxol on human leukemia HL-60 cells. Biochem. Pharmacol., *53:* 1587–1596, 1997.
9. Giannakakou, P., Robey, R., Fojo, T., and Blagosklonny, M. V. Low concentrations of paclitaxel induce cell type-dependent p53, p21 and $G_1/G_2$ arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. Oncogene, *20:* 3806–3813, 2001.
10. Torres, K., and Horwitz, S. B. Mechanisms of Taxol-induced cell death are concentration dependent. Cancer Res., *58:* 3620–3626, 1998.
11. He, L., Yang, C-P. H., and Horwitz, S. B. Mutations in b-tubulin map to domains involved in regulation of microtubule stability in epothilone-resistant cell lines. Mol. Cancer Ther., *1:* 3–10, 2001.
12. King, K. L., and Cidlowski, J. A. Cell cycle and apoptosis: common pathways to life and death. J. Cell Biochem., *58:* 175–180, 1995.
13. Li, X., and Nicklas, R. B. Mitotic forces control a cell-cycle checkpoint. Nature (Lond.), *373:* 630–632, 1995.
14. Speicher, L. A., Barone, L., and Tew, K. D. Combined antimicrotubule activity of estramustine and Taxol in human prostatic carcinoma cell lines. Cancer Res., *52:* 4433–4440, 1992.
15. Paoletti, A., Giocanti, N., Favaudon, V., and Bornens, M. Pulse treatment of interphasic HeLa cells with nanomolar doses of docetaxel affects centrosome organization and leads to catastrophic exit of mitosis. J. Cell Sci., *110:* 2403–2415, 1997.
16. Ngan, V. K., Bellman, K., Hill, B. T., Wilson, L., and Jordan, M. A. Mechanism of mitotic block and inhibition of cell proliferation by the semisynthetic *Vinca* alkaloids vinorelbine and its newer derivative vinflunine. Mol. Pharmacol., *60:* 225–232, 2001.
17. Sluder, G., Thompson, E. A., Miller, F. J., Hayes, J., and Rieder, C. L. The checkpoint control for anaphase onset does not monitor excess numbers of spindle poles or bipolar spindle symmetry. J. Cell Sci., *110:* 421–429, 1997.
18. Brinkley, B. R., and Goepfert, T. M. Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. Cell Motil. Cytoskeleton, *41:* 281–288, 1998.
19. Sena, G., Onado, C., Cappella, P., Montalenti, F., and Ubezio, P. Measuring the complexity of cell cycle arrest and killing of drugs: kinetics of phase-specific effects induced by Taxol. Cytometry, *37:* 113–124, 1999.
20. Iancu, C., Mistry, S. J., Arkin, S., and Atweh, G. F. Taxol and anti-stathmin therapy: a synergistic combination that targets the mitotic spindle. Cancer Res., *60:* 3537–3541, 2000.
21. Long, B. H., and Fairchild, C. R. Paclitaxel inhibits progression of mitotic cells to $G_1$ phase by interference with spindle formation without affecting other microtubule functions during anaphase and telephase. Cancer Res., *54:* 4355–4361, 1994.
22. Schimke, R. T., Kung, A., Sherwood, S. S., Sheridan, J., and Sharma, R. Life, death and genomic change in perturbed cell cycles. Philos. Trans. R. Soc. Lond. B Biol. Sci., *345:* 311–317, 1994.
23. Loeb, L. A. A mutator phenotype in cancer. Cancer Res., *61:* 3230–3239 2001.
24. Abal, M., Souto, A. A., Amat-Guerri, F., Acuna, A. U., Andreu, J. M., and Barasoain, I. Centrosome and spindle pole microtubules are main targets of a fluorescent taxoid inducing cell death. Cell Motil. Cytoskeleton, *49:* 1–15, 2001.
25. Keryer, G., Ris, H., and Borisy, G. G. Centriole distribution during tripolar mitosis in Chinese hamster ovary cells. J. Cell Biol., *98:* 2222–2229, 1984.